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ALTERATION OF PLANT MERISTEM FUNCTION BY MANIPULATION OF THE RETINOBLASTOMA-LIKE PLANT RRB GENE

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TITLE OF THE INVENTION

[0001] Alteration Of Plant Meristem Function By Manipulation Of The Retinoblastoma-Like Plant RRB Gene

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application is a continuation of U.S. Non-provisional Application No. 09/527,084 filed March 16, 2000, currently pending, which claims the benefit of U.S. Provisional Application No. 60/125,229 filed March 19, 1999, both of which are hereby incorporated herein by this reference. Reference is made to co-pending U.S. non-provisional application _____ , filed _____, docket number 102863, which is hereby incorporated herein by this reference, which is a divisional of U.S. Non-provisional Application No. 09/527,084 filed March 16, 2000, currently pending, which claims the benefit of U.S. Provisional Application No. 60/125,229 filed March 19, 1999. Reference is made to co-pending U.S. non-provisional application _____ , filed _____, docket number 102864 which is hereby incorporated herein by this reference, which is a continuation of U.S. Non-provisional Application No. 09/527,084 filed March 16, 2000, currently pending, which claims the benefit of U.S. Provisional Application No. 60/125,229 filed March 19, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] The United States Government has rights in this invention pursuant to Grant No. MCB9506985, between the National Science Foundation and the University of California, and Grant No. DE-FG03-88ER13882 between the U.S. Dept. of Energy and the University of California.

REFERENCE TO A "SEQUENCE LISTING", A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISC (SEE 37 CFR 1.52(E)(5))

[0004] Not Applicable

BACKGROUND OF THE INVENTION

Field of the Invention

[0005] The present invention relates generally to modulating the growth, differentiation and/or cell division of meristematic cells in a plant, more particularly modulating said cells via modulating RRB gene activity.

Description of Related Art

[0006] The ability to control the size of plants and plant tissues is an enormously valuable tool. For example, for many agricultural crops, increasing the size of a plant or of a specific tissues within a plant would be of obvious commercial value. Currently, most attempts to increase plant size or yield are accomplished through traditional or marker-assisted breeding programs. Such methods have, however, failed to provide methods to directly control the size of plants or plant tissues.

[0007] Most cell proliferation in plants occurs in tissues called meristematic tissue. Several types of meristematic tissue exist in plants, including the shoot apical meristem, which gives rise to all aerial parts of the plant, the root apical meristem, which establishes the root system, and the vascular meristem, which provides lateral growth of the plant. While several genes are known to alter meristem fate, and thereby plant development, the mechanism by which they function is poorly understood. The products of the *CLAVATA (CLV)* and *SHOOT MERISTEMLESS (STM1)* genes of Arabidopsis, for example, encoding a receptor-kinase and homeodomain protein, respectively, appear to work antagonistically in a shoot meristem maintenance pathway involved in the partitioning of the central-peripheral zone (CZ-PZ) of the meristem. Other genes, such as *ZWILLE (ZLL)* and *WUSCHEL (WUS)*, function early in embryonic development to specify the stem cells which will be maintained in the central zone of the shoot apical meristem. Other genes such as *MGOUN1(MGO1)* and *MGOUN2 (MGO2)* appear to function in the partitioning of cells from the PZ of the shoot apical meristem to leaf primordia or the inflorescence, often resulting in á fasciated meristem phenotype.

[0008] Plants containing mutations in the genes described above are defective in specific stages of meristem function and have well-characterized developmental phenotypes. As such, these genes are likely involved in the differentiation of meristematic cells, and are thus unlikely, by themselves, to provide tools to increase the size of plants or of plant tissues. Instead, it would be desirable to manipulate both the differentiation of meristematic cells as well as their growth and proliferation.

[0009] One potential method to alter the growth and/or proliferation of plant cells would be to modulate the activity of genes controlling these processes. For example, several groups have reported the cloning of at least a fragment of a Retinoblastoma-related protein in maize. *See, e.g.* Ach et al. (1997) Mol. Cell. Biol. 17:5077; Huntley et al. (1998) *Plant Mol. Biol.* 37:155; Grafi et al. (1996) *PNAS* 93:8962; Shen et al. (1994) Plant Mol. Biol. 26:1085; Xie et al. (1996) EMBO J 15:4900; and WO 97/47745. None of these studies, however, has investigated the function of RRB in proliferating, virus-free cells. Further, no studies have heretofore addressed the role of RRB in an intact plant. As well known to those of skill, only by examining the role of a protein in its normal environment, in an intact organism, can its true activity and/or function be determined.

[0010] Thus, the art lacks a good understanding of the function of RRB in plant cells and/or intact plants. Without this understanding, its use to control plant growth in an efficient manner is difficult if not impossible. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0011] This invention provides methods and compositions for altering the growth and differentiation of plant tissues. The invention is based on the discovery that, in plants, genetically altering the levels of Retinoblastoma-related gene (RRB) activity produces dramatic effects on the growth, proliferation, and differentiation of plant meristem. Altering the level of RRB activity in a plant tissue, therefore, can be used to specifically control the growth and/or differentiation of plant meristem, thereby controlling, *e.g.* the relative size and distribution of individual tissues in a plant.

[0012] In certain embodiments, this invention provides polynucleotides and polypeptides with plant RRB function. In one embodiment, the polynucleotide is as shown in SEQ ID NO:1 or SEQ ID NO: 9. In one embodiment, the polynucleotide encodes the polypeptide shown as SEQ ID NO:2, or fragments thereof. In a preferred embodiment, the polynucleotide encodes a full-length RRB protein. However, truncated forms of RRB proteins can be used as well. In addition, mutated forms of the RRB proteins can be used, *e.g.* as dominant negative forms.

[0013] This invention also provides transgenic plants comprising RRB polynucleotides. In preferred embodiments, the RRB polynucleotides are operably linked to a promoter, such as an inducible or tissue-specific promoter.

[0014] This invention also provides methods for inhibiting or enhancing the growth of plant cells, plant tissues, or entire plants. In preferred embodiments, RRB activity is enhanced or inhibited in a plant tissue by expressing a wild type, mutant, or truncated form of an RRB polynucleotide, or by expressing an inhibitor of RRB activity, *e.g.* a peptide that competitively binds RRB, thereby preventing its normal interaction with intracellular substrates.

[0015] The methods provided herein can also be used to alter the differentiation of a plant tissue. In preferred embodiments, the differentiation of a meristem is altered. For example, the present invention provides methods for modulating the RRB activity in an apical shoot meristem, thereby altering the size, organization, and/or differentiation of the meristem and, as a result, affecting the structure and/or number of, *e.g.*, a leaf primordium or an inflorescence bolt. Increasing or decreasing RRB activity can be effected in a plant, a plant tissue, or a plant cell by expressing a wild type, mutant, or truncated form of an RRB polynucleotide, or by expressing a peptide inhibitor of RRB activity. Such RRB polynucleotides are preferably linked to promoters such as a tissue-specific or an inducible promoter.

DEFINITIONS

[0016] A "nucleic acid" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. The term includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. With respect to a naturally occurring nucleic acid that is "isolated" from its natural environment, the nucleic acid is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. However, an "isolated" nucleic acid can refer to a recombinantly or synthetically produced nucleic acid, that is identical or altered from the naturally occurring nucleic acid sequence. In addition, an "isolated nucleic acid" can comprise naturally occurring nucleotides or can comprise any nucleotide derivative or analog, *e.g.* labeled nucleotides, that can be incorporated into a polynucleotide chain. Any aspect of the polynucleotide chain can be altered, such as the base, sugar, or phosphate backbone.

[0017] The term "promoter" refers to regions or sequence located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter that works in plants, *e.g.* CaMV 35S. A "tissue-specific promoter" is a promoter capable of initiating transcription in a certain tissue of a plant. A "tissue specific promoters" can comprise a naturally occurring promoter that drives the expression of a gene in one or more specific tissues, or can comprise modified, truncated, or otherwise modified derivatives of naturally occurring promoters, or can comprise a synthetic promoter with the desired properties. A "tissue specific promoter" can drive the expression of a gene in one or more tissues, and throughout the entire tissue or only in a subset of the tissue. In addition, a "tissue-specific promoter" can drive gene expression in a tissue throughout the life of a plant, or transiently at one or more times during the life of the plant.

[0018] The term "plant" includes whole plants, shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed

coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

[0019] "Recombinant" refers to a human manipulated polynucleotide or a copy or complement of a human manipulated polynucleotide. For instance, a recombinant expression cassette comprising a promoter operably linked to a second polynucleotide may include a promoter that is heterologous to the second polynucleotide as the result of human manipulation (*e.g.*, by methods described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)) of an isolated nucleic acid comprising the expression cassette. In another example, a recombinant expression cassette may comprise polynucleotides combined in such a way that the polynucleotides are extremely unlikely to be found in nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second polynucleotide. One of skill will recognize that polynucleotides can be manipulated in many ways and are not limited to the examples above.

[0020] As used herein, the term "RRB polynucleotide" refers to any polynucleotide encoding a polypeptide with RRB activity, and which encodes a polypeptide with at least about 50% sequence identity to the exemplified sequences provided herein. The RRB polypeptides encoded by RRB polynucleotides have at least about 50%, 60%, 70%, 80%, 90% or higher sequence identity at the deduced amino acid level relative to the exemplary RRB polynucleotide sequences provided herein. "RRB polynucleotide" includes reference to nucleic acids of at least about 20, 30, 40, or 50 nucleotides in length, more preferably about

100, 200, 500, 1000, 2000, 5000 or more nucleotides. Thus, an "RRB polynucleotide" can be an RRB gene or a subsequence thereof.

[0021] "RRB activity" refers to one or more biochemical or genetic properties of an RRB polynucleotide or polypeptide. For example, when expressed in cells or tissues of a plant, an RRB polynucleotide can affect the growth, proliferation, and/or differentiation of plant cells and tissues, resulting in the phenotypes described herein. In addition, RRB can bind to a number of heterologous proteins, such as E2F, D-type cyclins, or viral proteins such as large-T antigen or E1A, or the geminivirus protein RepA. Often, such proteins will bind RRB through an LXCXE motif. Accordingly, RRB activity can be assessed based on binding to any LXCXE-motif containing polypeptide. Any of these activities, *inter alia*, can be monitored or modified according to the present invention.

[0022] An "inhibitor of RRB activity", as used herein, refers to any material that results in the decrease of RRB activity. Such molecules can include expressible forms of RRB polynucleotides, such as antisense RRB polynucleotides, RRB polynucleotides used to inhibit by co-suppression, dominant-negative forms of RRB such as truncated or mutated forms of RRB, as well as other expressible inhibitors such as peptide inhibitors of RRB or anti-RRB ribozymes. In addition, an "inhibitor of RRB activity" can include any material that can be used to decrease RRB activity, such as molecules that inhibit the activity, expression, or stability of RRB polynucleotides or polypeptides.

[0023] Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection.

When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, *e.g.*, the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

[0024] The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence, which has substantial sequence or subsequence complementarity when the test sequence has substantial identity to a reference sequence.

[0025] One of skill in the art will recognize that two polypeptides can also be "substantially identical" if the two polypeptides are immunologically similar. Thus, overall protein structure may be similar while the primary structure of the two polypeptides display significant variation. Therefore a method to measure whether two polypeptides are substantially identical involves measuring the binding of monoclonal or polyclonal antibodies to each polypeptide. Two polypeptides are substantially identical if the antibodies specific for

a first polypeptide bind to a second polypeptide with an affinity of at least one third of the affinity for the first polypeptide.

[0026] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0027] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

[0028] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The

method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

[0029] Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl.*

Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0030] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0031] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0032] As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single

amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

[0033] The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

[0034] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

[0035] The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA).

[0036] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of

nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, highly stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. Low stringency conditions are generally selected to be about 15-30 °C below the T_m. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 time background hybridization.

[0037] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

[0038] In the present invention, genomic DNA or cDNA comprising RRB nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed here. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a

temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. In preferred embodiments, stringent hybridization conditions for screening cDNA libraries and/or for Southern blot hybridizations include:

Hybridization at 55°C in the following:

0.75M NaCl
5mM EDTA pH 8
0.15M Tris HCl pH 8
2.75mM tetra sodium pyrophospate
0.1% Ficoll
0.1% polyvinyl pyrrolidone
0.1% BSA
10% Dextran sulphate
0.1% SDS
0.05 mg/ml herring sperm DNA

[0039] Washing in 2X SSC, 0.1% SDS at 55°C, using, e.g. an RRB cDNA as a probe for hybridization.

[0040] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cased, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice

background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

[0041] A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, an RNA gel or DNA gel blot hybridization analysis.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

[0042] Not Applicable

DETAILED DESCRIPTION OF THE INVENTION

[0043] This invention provides methods of directly controlling the size and/or differentiation state of plants, plant tissues, or plant cells. This invention is based on the surprising discovery that, in intact plants, alterations in RRB levels dramatically affect the growth, organization, as well as differentiation of specific tissues. By specifically increasing or decreasing the level of RRB in cells within an intact plant, therefore, it is possible to increase or decrease the size of the tissue or plant comprising the cells. When directed to specific tissues within a plant, it is thus possible to specifically and controllably alter the growth and/or differentiation of the tissue.

I. RRB

[0044] Any of a number of RRB sequences can be used in the present invention. RRB sequences can be used from any monocotyledonous or dicotyledonous plant, such as *Arabidopsis*, *Zea mays*, *Chenopodium*, and tobacco. In addition, RRB homologs from animals, such as from mammals, fish, birds, insects, etc. can be used. In preferred embodiments, an RRB nucleotide sequence will be used that will hybridize, under low to

moderate stringency, to SEQ ID NO:1, 3, 5, 7, or 9, or which is substantially identical to all or part of SEQ ID NO:1, 3, 5, 7, or 9. Also preferred is the use of RRB polypeptides substantially similar to all or part of SEQ ID NO:2, 4, 6, or 8. The present invention can be used with full-length, truncated, wild type, or mutated forms of RRB, as described *infra*.

[0045] Typically, the RRB sequences will include one or more functional domains characteristic of RRB sequences, such as the A or B pocket, one or more protein or protein-motif binding domains, *e.g.* an LXCXE motif binding domain, and phosphorylation sites. In addition, the N-terminal 130 amino acids, or 383 5' nucleotides, of the *Arabidopsis* sequence, which are not found in *Zea mays*, can be used. Such *Arabidopsis*-specific sequences can readily be identified by comparing an *Arabidopsis* sequence, *e.g.* SEQ ID NO:1, with, *e.g.* a *Zea mays* sequence as shown in SEQ ID NO:3, 5,or 7. RRB sequences can be isolated from any natural source, can be derived from a natural source, *i.e.* a mutated or truncated derivative, or can be synthesized *de novo*. Methods for purifying, mutating, and recombinantly altering nucleic acids are well known in the art, and can be found in any of a multitude of guides, such as Sambrook *et al.*, (1989) and Ausubel *et al.* (1999).

II. Altering RRB expression and/or activity in plant tissues

[0046] The present invention can be used to alter the growth, organization, and/or differentiation of any of a number of plant tissues. Typically, the tissues will comprise meristematic tissue, including root meristem, shoot apical meristem, vascular meristem, or endosperm. In certain embodiments, RRB activity may be modulated in non-meristematic tissue, *e.g.* to affect the differentiation of the tissue or, *e.g.* to promote proliferation in normally non-proliferating cells. Accordingly, the present methods can be used to affect the growth and/or differentiation of any part of a plant, including roots, stems, leaves, flowers, seed, fruit, tubers etc., as well as any structure within one of these parts, *e.g.* bracts, sepals, petals, stamens, carpels, anthers, ovules, embryos, endosperm, and seed coat).

[0047] Any of these tissues can be targeted individually or in combination, *e.g.* using one or more tissue specific promoters such as leaf-specific promoters, flower meristem-specific promoters, endosperm-specific promoters, root-specific promoters, etc. Also, the tissues can be targeted at all times during the life of the plant, *e.g.* using a constitutive promoter, or transiently, *e.g.* using a transiently active or an inducible promoter. It will be appreciated that, *e.g.* using multiple expression constructs, RRB activity can be simultaneously increased in one tissue and decreased in another in a single plant, thereby altering the relative sizes of the tissues within a plant. For commercial crops, such methods would allow the relative increase in the yield of valuable tissues, and the decrease in size of unwanted tissues.

[0048] These methods can be used to enhance and/or inhibit the growth and differentiation of plant cells. Further, we have discovered a relationship between the amount of increase or decrease in the level of RRB activity and the degree to which growth, organization, and/or differentiation is affected. For example, in a transgenic plant with an RRB polynucleotide under the control of an inducible promoter, adding a small amount of the inducing agent results in a mild effect on growth, organization, and/or differentiation, whereas adding a substantial amount of the agent results in dramatic changes in the rate or level of growth, organization and/or differentiation. Accordingly, the present invention can be used to alter the degree to which a plant tissue grows, organizes, and/or differentiates, *e.g.* by using a variable amount of an inducing agent or by using promoters of various strengths.

[0049] In certain embodiments, the level of RRB activity will be altered alone, *i.e.* no other cellular moieties will be manipulated. In other embodiment, however, RRB levels can be altered in conjunction with other cellular components. For example, other regulators of cell growth, cell proliferation, or cellular differentiation may be altered to enhance or attenuate the effects of the altered RRB levels. In certain embodiments, genes involved in meristem formation and/or differentiation may be used, *e.g.* CLV, STM1, ZLL, WUS, MGO1, or MGO2. For example, a gene promoting meristem formation may be used to increase the amount of meristem, which can be increased further by, *e.g.* modulating the levels of RRB in

the meristem. Finally, the enlarged meristem can subsequently be induced to differentiate by further altering RRB activity in the cells.

A. Increasing RRB activity or *RRB* gene expression

[0050] Any of a number of means well known in the art can be used to increase RRB activity in plants. Increased RRB activity can be used to, *e.g.* modulate the growth of plant tissues, modulate the organization of plant tissues, and/or modulate the differentiation of the tissues. In a preferred embodiment, increasing RRB activity in cells within a plant or a plant tissue results in a decrease in the size of the plant or plant tissue. Any organ can be targeted, such as shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit. Alternatively, one or more RRB genes can be expressed constitutively (*e.g.*, using a constitutive promoter).

1. Increasing expression of RRB polynucleotides

[0051] Isolated sequences prepared as described herein can be used to introduce expression of a particular *RRB* nucleic acid to increase gene expression using methods well known to those of skill in the art. Preparation of suitable constructs and means for introducing them into plants are described below.

[0052] One of skill will recognize that the polypeptides encoded by the genes of the invention, like other proteins, have different domains that perform different functions. Thus, the gene sequences need not be full length, so long as the desired functional domain of the protein is expressed. For example, RRB can bind to various proteins, such as E2F, D-type cyclins, E1A, large T-antigen, and other viral proteins, and has multiple conserved domains, such as the A and B pocket domains and conserved phosphorylation sites. Any of these binding sites or conserved regions may be used in the present invention.

[0053] Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described in detail below. For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

[0054] In certain embodiments, modified forms of RRB will be used that have increased RRB activity in vivo. For example, creating forms of RRB that cannot be inhibited by phosphorylation will create a hyperactive form of RRB. Additional hyperactive forms can be readily identified, *e.g.* by screening for modified forms of RRB with an enhanced ability to inhibit the cell cycle or to promote differentiation.

[0055] RRB polynucleotide expression can be increased throughout a plant, in one or more tissues or cells of a plant, and constitutively or transiently. Such expression patterns can be achieved using any of a variety of promoters, including endogenous RRB promoters, heterologous promoters, constitutive promoters, tissue-specific promoters, and inducible promoters.

2. Modification of endogenous *RRB* genes to increase RRB activity

[0056] In certain embodiments of this invention, endogenous RRB will be targeted for modification. Methods for introducing genetic mutations into plant genes and selecting plants with desired traits are well known. For instance, seeds or other plant material can be treated with a mutagenic chemical substance, according to standard techniques. Such chemical substances include, but are not limited to, the following: diethyl sulfate, ethylene imine, ethyl methanesulfonate and N-nitroso-N-ethylurea. Alternatively, ionizing radiation from sources such as, X-rays or gamma rays can be used.

[0057] Alternatively, homologous recombination can be used to induce targeted gene modifications by specifically targeting the *RRB* gene *in vivo* (*see, generally*, Grewal and Klar, *Genetics* 146: 1221-1238 (1997) and Xu *et al.*, *Genes Dev.* 10: 2411-2422 (1996)). Homologous recombination has been demonstrated in plants (Puchta *et al.*, *Experientia* 50: 277-284 (1994), Swoboda *et al.*, *EMBO J.* 13: 484-489 (1994); Offringa *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 7346-7350 (1993); and Kempin *et al. Nature* 389:802-803 (1997)).

[0058] In applying homologous recombination technology to the genes of the invention, mutations in selected portions of an *RRB* gene sequence (including 5' upstream, 3' downstream, and intragenic regions) such as those disclosed here are made *in vitro* and then introduced into the desired plant using standard techniques. Since the efficiency of homologous recombination is known to be dependent on the vectors used, use of dicistronic gene targeting vectors as described by Mountford *et al., Proc. Natl. Acad. Sci. USA* 91: 4303-4307 (1994); and Vaulont *et al., Transgenic Res.* 4: 247-255 (1995) are conveniently used to increase the efficiency of selecting for altered *RRB* gene expression in transgenic plants. The mutated gene will interact with the target wild-type gene in such a way that homologous recombination and targeted replacement of the wild-type gene will occur in transgenic plant cells, resulting in increased RRB activity.

[0059] Alternatively, oligonucleotides composed of a contiguous stretch of RNA and DNA residues in a duplex conformation with double hairpin caps on the ends can be used. The RNA/DNA sequence is designed to align with the sequence of the target *RRB* gene and to contain the desired nucleotide change. Introduction of the chimeric oligonucleotide on an extrachromosomal T-DNA plasmid results in efficient and specific RRB gene conversion directed by chimeric molecules in a small number of transformed plant cells. This method is described in Cole-Strauss *et al.*, *Science* 273:1386-1389 (1996) and Yoon *et al. Proc. Natl. Acad. Sci. USA* 93: 2071-2076 (1996).

3. Other means for increasing RRB activity

[0060] One method to increase *RRB* expression is to use "activation mutagenesis" (*see, e.g.* Hiyashi *et al. Science* 258:1350-1353 (1992)). In this method an endogenous RRB gene can be modified to be expressed constitutively, ectopically, or excessively by insertion of T-DNA sequences that contain strong/constitutive promoters upstream of the endogenous RRB gene. As explained below, preparation of transgenic plants overexpressing RRB can also be used to increase RRB expression. Activation mutagenesis of the endogenous RRB gene will give the same effect as overexpression of the transgenic RRB nucleic acid in transgenic plants. Alternatively, an endogenous gene encoding an enhancer of RRB activity or expression of the endogenous RRB gene can be modified to be expressed by insertion of T-DNA sequences in a similar manner and RRB activity can be increased.

[0061] Another strategy to increase RRB expression can involve the use of dominant hyperactive mutants of RRB by expressing modified RRB transgenes. For example expression of modified RRB with a defective domain that is important for interaction with a negative regulator of RRB activity can be used to generate dominant hyperactive RRB proteins. Alternatively, expression of truncated RRB proteins which have only a domain that interacts with a negative regulator can titrate the negative regulator and thereby increase endogenous RRB activity. Use of dominant mutants to hyperactivate target genes is described, *e.g.*, in Mizukami *et al. Plant Cell* 8:831-845 (1996).

B. Inhibition of RRB activity or gene expression

[0062] As explained above, RRB activity is important in controlling the growth and differentiation of cells. Inhibition of RRB gene expression activity can be used, for instance, to alter cell growth and/or proliferation, to modulate tissue organization, and/or to modulate differentiation of cells within a tissue or plant. In a preferred embodiment, decreasing RRB activity in cells of a plant or a plant tissue results in an increase in the size of the plant or plant tissue. In particular, targeted expression of RRB nucleic acids that inhibit endogenous gene expression (*e.g.*, antisense or co-suppression) can be used.

1. Inhibition of RRB gene expression

[0063] The nucleic acid sequences disclosed herein can be used to design nucleic acids useful in a number of methods to inhibit RRB or related gene expression in plants. For instance, antisense technology can be conveniently used. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been suggested that antisense suppression can act at all levels of gene regulation including suppression of RNA translation (*see*, Bourque *Plant Sci. (Limerick)* 105: 125-149 (1995); Pantopoulos In Progress in Nucleic Acid Research and Molecular Biology, Vol. 48. Cohn, W. E. and K. Moldave (Ed.). Academic Press, Inc.: San Diego, California, USA; London, England, UK. pp. 181-238; Heiser *et al. Plant Sci. (Shannon)* 127: 61-69 (1997)) and by preventing the accumulation of mRNA which encodes the protein of interest, *(see,* Baulcombe *Plant Mol. Bio.* 32:79-88 (1996); Prins and Goldbach *Arch. Virol.* 141: 2259-2276 (1996); Metzlaff *et al. Cell* 88: 845-854 (1997), Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340).

[0064] The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous RRB gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting identity or substantial identity to the target gene.

[0065] For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and about full length nucleotides should be used, though a sequence of at least

about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of about 500 to about 3500 nucleotides is especially preferred.

[0066] A number of gene regions can be targeted to suppress RRB gene expression. The targets can include, for instance, the coding regions, introns, sequences from exon/intron junctions, 5' or 3' untranslated regions, and the like.

[0067] Another well-known method of suppression is sense co-suppression. Introduction of nucleic acid configured in the sense orientation has been recently shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes (*see,* Assaad *et al. Plant Mol. Bio.* 22: 1067-1085 (1993); Flavell *Proc. Natl. Acad. Sci. USA* 91: 3490-3496 (1994); Stam *et al. Annals Bot.* 79: 3-12 (1997); Napoli et al., *The Plant Cell* 2:279-289 (1990); and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184).

[0068] The suppressive effect may occur where the introduced sequence contains no coding sequence *per se*, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting identity or substantial identity.

[0069] For co-suppression, the introduced sequence, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants that over-express the introduced sequence. A higher identity in a sequence shorter than full-length compensates for a longer, less identical sequence. Furthermore, the introduced

sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used. In addition, the same gene regions noted for antisense regulation can be targeted using co-suppression technologies.

[0070] Oligonucleotide-based triple-helix formation can also be used to disrupt *RRB* gene expression. Triplex DNA can inhibit DNA transcription and replication, generate site-specific mutations, cleave DNA, and induce homologous recombination (*see, e.g.*, Havre and Glazer *J. Virology* 67:7324-7331 (1993); Scanlon *et al. FASEB J.* 9:1288-1296 (1995); Giovannangeli *et al. Biochemistry* 35:10539-10548 (1996); Chan and Glazer *J. Mol. Medicine (Berlin)* 75: 267-282 (1997)). Triple helix DNAs can be used to target the same sequences identified for antisense regulation.

[0071] Catalytic RNA molecules or ribozymes can also be used to inhibit expression of RRB genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. Thus, ribozymes can be used to target the same sequences identified for antisense regulation.

[0072] A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs that are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Zhao and Pick, *Nature* 365:448-451 (1993); Eastham and Ahlering, *J. Urology* 156:1186-1188 (1996); Sokol and

Murray, *Transgenic Res.* 5:363-371 (1996); Sun *et al., Mol. Biotechnology* 7:241-251 (1997); and Haseloff *et al., Nature*, 334:585-591 (1988).

2. Modification of endogenous *RRB* genes

[0073] Methods for introducing genetic mutations described above can also be used to select for plants with decreased *RRB* expression.

3. Other methods for inhibiting RRB activity

[0074] RRB activity may be modulated by eliminating the proteins that are required for *RRB* cell-specific gene expression. Thus, expression of regulatory proteins and/or the sequences that control *RRB* gene expression can be modulated using the methods described here.

[0075] Another strategy is to inhibit the ability of a RRB protein to interact with itself or with other proteins. This can be achieved, for instance, using antibodies specific to RRB. In this method cell-specific expression of RRB-specific antibodies is used to inactivate functional domains through antibody:antigen recognition (*see*, Hupp *et al., Cell* 83:237-245 (1995)). Interference of activity of a RRB interacting protein(s) can be applied in a similar fashion.

[0076] Alternatively, dominant negative mutants of RRB can be prepared by expressing a transgene that encodes a truncated RRB protein. Use of dominant negative mutants to inactivate target genes in transgenic plants is described in Mizukami *et al., Plant Cell* 8:831-845 (1996). In a preferred embodiment, an RRB polypeptide with a mutation that prevents binding of RRB to heterologous proteins, *e.g.* a mutation in a conserved cysteine residue (corresponding to C706 of human RB), is expressed in a cell. With respect to the Arabidopsis cDNA shown as SEQ ID NO:1, the alteration comprises a cysteine to phenylalanine substitution, resulting from a G to T change at position 2363 bp. In particularly preferred embodiments, such mutated or truncated RRB proteins are expressed at a level at least as high as that of the endogenous RRB protein.

[0077] Another approach to inhibit RRB activity is through the use of peptide inhibitors of RRB activity. Such inhibitors may be derived from naturally occurring proteins, *e.g.* RRB binding proteins. For example, a fragment of E2F that competitively binds RRB and prevents it from binding to full length E2F may be expressed in a cell. Also, a peptide include an LXCXE motif can be used, thereby competitively blocking the binding of proteins such as D-type cyclins to RRB. However, any peptide with the ability to inhibit RRB activity, by interacting directly with RRB itself or with a substrate of RRB, can be used. Such peptides can be easily identified, for example, by generating a library of peptide molecules and screening the library for peptides with the ability to bind to and/or inhibit RRB *in vitro* or *in vivo*.

[0078] In certain embodiments, a non-peptide inhibitor of RRB can be used. Such inhibitors can be any molecule or treatment that reduces RRB activity in a cell. Such molecules can include organic compounds including nucleic acids, nucleotides, amino acids, carbohydrates, fats, waxes, hormones, etc., or any inorganic compounds. Any compound can be screened for the ability to bind to and/or inhibit RRB activity, in vitro or in vivo. In addition, any non-molecular treatment, *e.g.*. temperature, electromagnetic radiation, motion, etc. that affects RRB activity can be employed.

III. Isolation and manipulation of RRB polynucleotides and polypeptides

A. Purification of RRB polypeptides

[0079] Either naturally occurring or recombinant RRB polypeptides can be purified for use in functional assays, *e.g.* protein binding assays. Naturally occurring RRB polypeptides can be purified, *e.g.*, from plant tissue and any other source of a RRB homolog. Recombinant RRB polypeptides can be purified from any suitable expression system.

[0080] The RRB polypeptides may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate;

column chromatography, immunopurification methods, and others (see, *e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*).

[0081] A number of procedures can be employed when recombinant RRB polypeptides are being purified. For example, proteins having established molecular adhesion properties can be reversible fused to the RRB polypeptides. With the appropriate ligand, the RRB polypeptides can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally the RRB polypeptides could be purified using immunoaffinity columns.

B. Isolation of RRB nucleic acids

[0082] Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998).

[0083] The isolation of RRB nucleic acids may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, *e.g.* using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as leaves, and a cDNA library which contains a RRB gene transcript is

prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which RRB genes or homologs are expressed.

[0084] The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned RRB gene disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Alternatively, antibodies raised against a RRB polypeptide can be used to screen an mRNA expression library.

[0085] Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of RRB genes directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR see *PCR Protocols: A Guide to Methods and Applications*. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), *Academic Press*, San Diego (1990). Appropriate primers and probes for identifying RRB sequences from plant tissues are generated from comparisons of the sequences provided herein (*e.g.* SEQ ID NO: 1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, etc.).

[0086] Polynucleotides may also be synthesized by well-known techniques, as described in the technical literature. See, *e.g.*, Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

C. Preparation of recombinant vectors

[0087] To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising et al. *Ann. Rev. Genet.* 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

[0088] For example, for overexpression, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S and 19S transcription initiation regions; the full-length FMV transcript promoter (Gowda *et al.*, *J Cell Biochem* 13D:301;the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumafaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such promoters and others are described, *e.g.* in U.S. Patent No. 5,880,330. Such genes include for example, *ACT11* from *Arabidopsis* (Huang *et al. Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al. Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No. X15596, Martinez *et al. J. Mol. Biol* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)).

[0089] Alternatively, the plant promoter may direct expression of RRB nucleic acid in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (*i.e.* inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include

anaerobic conditions, elevated temperature, the presence of light, or sprayed with chemicals/hormones. Numerous inducible promoters are known in the art, any of which can be used in the present invention. Such promoters include the yeast metallothionine promoter, which is activated by copper ions (*see, e.g.* Mett *et al.* (1993) PNAS 90:4567), the dexamethasone-responsive promoter, In2-1 and In2-2, which are activated by substituted benzenesulfonamides, and GRE regulatory sequences, which are glucocorticoid-responsive.

[0090] Tissue-specific promoters can be inducible. Similarly, tissue-specific promoters may only promote transcription within a certain time frame of developmental stage within that tissue. Other tissue specific promoters may be active throughout the life cycle of a particular tissue. One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

[0091] A number of tissue-specific promoters can also be used in the invention. For instance, promoters that direct expression of nucleic acids in leaves, roots or flowers are useful for the growth, proliferation, and/or differentiation of those organs. For expression of a RRB polynucleotide in the aerial vegetative organs of a plant, photosynthetic organ-specific promoters, such as the *RBCS* promoter (Khoudi, *et al.*, *Gene* 197:343, 1997), can be used. Root-specific expression of RRB polynucleotides can be achieved under the control of the root-specific *ANR1* promoter (Zhang & Forde, *Science*, 279:407, 1998). Other suitable tissue specific promoters include the cdc2a and cyc07 promoters, the histone promoter, the cinnamyl alcohol dehydrogenase (CAD) promoter, the mustard CHS1 promoter, the bean grp 1.8 promoter, the PAL1 promoter, the chalcone synthase A promoter, the UFO promoter, and others. In preferred embodiments, a promoter will be used that drives RBB expression specifically in a meristem. In preferred embodiments, an RRB promoter will be used. For example, the RRB promoter shown in SEQ ID NO:9 (*e.g.* approximately base pairs 1-543) can be used to drive expression of operably linked sequences in meristematic and other tissues in Arabidopsis or any type of plant.

[0092] In addition, the promoter shown in SEQ ID NO:9 (*e.g.* approximately base pairs 1 to 543 or, *e.g.* 1-1000) can be used to drive the expression of heterologous genes in meristematic tissue. RRB promoters can be used to drive the expression of any heterologous gene whose expression in meristematic tissue is desired. For example, cell cycle-related genes such as cyclins, Cdks, E2F, DP, p53, Cdc25, CKIs, or any derivative or variation thereof, can be used, as can developmental genes such as CLV, STM1, ZLL, WUS, MGO1, or MGO2.

[0093] If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

[0094] The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

IV. Production of transgenic plants

[0095] DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment.

[0096] Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al. Embo. J.* 3:2717-2722 (1984). Electroporation

techniques are described in Fromm *et al. Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al. Nature* 327:70-73 (1987).

[0097] Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch *et al. Science* 233:496-498 (1984), and Fraley *et al. Proc. Natl. Acad. Sci. USA* 80:4803 (1983) and *Gene Transfer to Plants*, Potrykus, ed. (Springer-Verlag, Berlin 1995).

[0098] Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as increased seed mass. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. *Ann. Rev. of Plant Phys.* 38:467-486 (1987).

[0099] The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca,*

Linum, Lolium, Lupinus, Lycopersicon, Malus, Manihot, Majorana, Medicago, Nicotiana, Olea, Oryza, Panieum, Pannesetum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Prunus, Raphanus, Ricinus, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Triticum, Vicia, Vitis, Vigna, and *Zea*.

[0100] One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0101] Using known procedures one of skill can screen for plants of the invention by detecting the increase or decrease of RRB mRNA or protein in transgenic plants. Means for detecting and quantitating mRNAs or proteins are well known in the art.

EXAMPLES

[0102] The full-length *Arabidopsis thaliana* Rb *(AtRRB)* cDNA was cloned into plant expression cassettes behind a dexamethasone-inducible promoter (DEX-inducible plasmid pTA7002) to manipulate ectopic expression *(AtRRB-OE)* of the *AtRRB* gene. A mutated version of the *AtRRB* cDNA containing a cystidine to phenylalanine substitution resulting from a G to T change at position 2363 bp was also cloned into a plant expression cassette *(AtRRBcys-OE)*. By analogy to metazoan Rb-like proteins, the protein encoded by *AtRRBcys* would be altered in structure and function and is predicted to act as a dominant negative mutation when ectopically expressed.

[0103] Numerous phenotypic alterations were observed in transgenic lines, including enlargement of the inflorescence bolt, or formation of multiple meristems at the shoot apex. Other phenotypic alterations include delayed leaf emergence, altered leaf morphology (with regard to degree of development, shape and fused organs), or terminal flower formation. A subset of transgenic lines show a complete loss of shoot and root apical meristem activity.

Our results document a key role for the product of the plant *RRB* gene in meristem differentiation, organization, and the meristematic cell cycle. The plant *RRB* gene is therefore a key target for functional manipulations to alter cell cycle regulation, apportioning of cells to primordia, and cellular differentiation in shoot and root apical meristems.

[0104] Numerous phenotypes were observed in most of the transgenic lines in the uninduced condition, *i.e.* in the absence of dexamethasone (the DEX--inducible promoter system is known to be "leaky" *in vivo).* Phenotypes were typically enhanced following induction of the promoter with dexamethasone. These results demonstrate that we can modify expression of the transgenes and phenotypic responses. Of the different phenotypes obtained in the *DEX- AtRRB-OE* and DEX- *AtRRBcys-OE* transgenic lines, the most penetrant phenotypes observed are in the impaired ability of the meristem to (1) generate leaf primordia and (2) to maintain a proper size and/or organization, as observed by severely delayed leaf emergence and fasciation of the inflorescence bolt, respectively. These phenotypes are reminiscent o *Arabidopsis thaliana mgo* mutants, which have a similar phenotype including delayed emergence of leaves and an enlarged shoot apical meristem, observed as fasciation. These results suggest that one function of *AtRRB* is the removal of cells from the PZ of the shoot apical meristem and in the differentiation of the leaf primordia.

[0105] Other phenotypes which occur in the most transgenic lines to different degrees of penetrance include (1) the development of adventitious meristems and/or splitting of the shoot apical meristem, (2) the production of leaves which are altered in shape or are fused, and (3) the inability of the inflorescence meristem to maintain a population of undifferentiated cells which results in the production of a terminal flower. These phenotypes support the conclusion that defects in shoot apical and inflorescence meristem formation, maintenance or function are obtained by manipulating *AtRRB* expression *in vivo.*

[0106] Transgenic lines expressing *DEX- AtRRB-OE* that have severe phenotypes in the uninduced condition showed an extreme phenotype when the DEX-inducible promoter was activated. Shoot and root apical meristem function was completely attenuated. Primary

and secondary roots and primary leaves did not form, and the plants died with expanded but small cotyledons. This severe phenotype showed a penetrance of 100% in a population of hemizygous and homozygous individuals of two lines and slightly less in a third line. The complete loss of shoot and root meristem function in these lines following DEX induction confirms that *AtRRB* has a key role in meristem formation and maintenance, including control of cell cycle activity within proliferating populations of meristem cells and/or organ primordia differentiation.

[0107] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above may be used in various combinations. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.